Optimizacija uvjeta za trodimenzionalni rast hrskavice in vitro

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„OPTIMIZATION OF CONDITIONS FOR *IN VITRO* THREE-DIMENSIONAL CARTILAGE GROWTH"

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OPTIMIZACIJA UVJETA ZA TRODIMENZIONALNI RAST HRSKAVICE *IN VITRO*

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Artikularna hrskavica je tkivo koje nema sposobnost spontane regeneracije. Tkivno inženjerstvo, kao novo područje regenerativne medicine, nudi potencijalna učinkovita rješenja u liječenju ozljeda i oštećenja artikularne hrskavice. Cilj ovoga istraživanja bio je optimizirati uvjete za trodimenzionalni uzgoj hrskavice *in vitro*. U istraživanju su korištena tri različita tipa staničnih kultura: hondrociti, mezenhimske matične stanice iz koštane srži (hBMSCs) te kombinacija hondrocita i hBMSCs u omjeru 2:1. Stanice su uzgajane u diferencijacijskom mediju, inkorporirane u RADA peptidni hidrogel, u normoksičnim (20% O_2) ili hipoksičnim (5% O_2) uvjetima. Dexametazon, jedna od komponenti diferencijacijskoga medija, bila je dodana u medij ili inkorporirana sa stanicama u peptidnom hidrogelu. Nakon 19 dana metodom kvantitativnog PCR-a ispitana je razina ekspresije gena *SOX9* i *ACAN*, karakterističnih za hrskavicu. Među korištenim tipovima stanica hondrociti su pokazali najviši, a hBMSCs najniži hondrogeni potencijal. Hipoksija je značajno poboljšala hondrogenezu jedino u slučaju hondrocita, dok izvor dexametazona nije bitno utjecao na razinu ekspresije gena *SOX9* i *ACAN*.

BASIC DOCUMENTATION CARD

OPTIMIZATION OF CONDITIONS FOR *IN VITRO* THREE-DIMENSIONAL CARTILAGE GROWTH

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Articular cartilage is a tissue that shows no capacity for effective spontaneous regeneration. Tissue engineering, as a new important field of regenerative medicine, emerges as potential effective solution for treatment of articular cartilage injuries and defects. The aim of this study was to optimize conditions for 3D *in vitro* chondrogenesis. In the experiment, three different cell culture types were used: chondrocytes, human bone marrow mesenchymal stem cells (hBMSCs) and combination of chondrocytes and hBMSCs in 2:1 ratio. Cells were grown in a chondrogenic medium, incorporated in a peptide hydrogel RADA, in either normoxic (20% O_2) or hypoxic (5% O_2) conditions. Dexamethasone, one of the chondrogenic medium components, was added in a differentiation medium or it was incorporated with cells within a peptide hydrogel. After 19 days, expression levels of two cartilage marker genes, *SOX9* and *ACAN*, were evaluated by quantitative PCR. Chondrocytes showed the highest and hBMSCs the lowest chondrogenic potential among used cell types. Hypoxia enhanced chondrogenesis significantly only in the case of chondrocytes, while dexamethasone source did not considerably influence on expression levels of *SOX* and *ACAN* genes.

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1. INTRODUCTION

1.1. Cartilage tissue biology

Cartilage is a form of tough and flexible connective tissue composed of sparsely distributed chondrocytes embedded within a dense extracellular matrix (ECM) characterized by high amount of glycosaminoglycans and proteoglycans which interact with collagen and elastic fibers. Chondrocytes are essential for production and maintenance of ECM components and are located in the matrix cavities called lacunae (Mescher, 2013).

Cartilage is avascular and receives nutrients solely by diffusion from capillaries in adjacent connective tissue (perichondrium) or, in the case of articular cartilage, from synovial fluid (Mescher, 2013; Horvai, 2011). Large blood vessels can traverse cartilage in order to supply other tissues, but these vessels release few nutrients to the cartilage. Cartilage also lacks lymphatic vessels and nerves.

The firm structure of ECM contributes to its resiliency which allows cartilage to bear mechanical stresses without permanent distortion. It serves as shock-adsorbing and sliding surface within joints and facilitates bone movements. In some parts of the body, cartilage supports soft tissues like those in the respiratory tract, nose and ears. It also plays important role in development and growth of long bones, both before and after birth (Mescher, 2013).

Based on differences in matrix composition, location in the body and functional requirements, three main types of cartilage can be distinguished: hyaline cartilage, elastic cartilage and fibrocartilage.

Hyaline cartilage (gr. *hyalos* = glassy) is the predominant type in the human body. In adults, hyaline cartilage is located in the articular surfaces of movable joints, the ventral aspects of the ribs, in the walls of larger respiratory passages (nose, larynx, trachea, bronchi). It also exists in the epiphyseal plates (growth plates) of long bones allowing longitudinal bone growth. During skeletal development, it forms temporary skeleton that is gradually replaced by bone.

Elastic cartilage is present in the auricle of the ear, the walls of the external auditory canals, the eustachian tube, the epiglottis and parts of the larynx.

Fibrocartilage comes in various forms, but it is mostly composed of hyaline cartilage and dense connective tissue with gradual transitions between these tissues. It exists in invertebral discs, the symphysis pubis and in the junctions between large tendons and articular cartilage in large joints (Meyer et al, 2006).

1.2. Articular cartilage

Articular cartilage is a form of hyaline cartilage, located in the diarthrodial (synovial) joints and best characterized of cartilage subtypes (Horvai, 2011). Because of its smooth and lubricated surface, articular cartilage has impressive load-bearing capacity and provides almost frictionless articulation of joint surfaces. Moreover, articular cartilage distributes the loads over a larger contact area which minimizes contact stresses. It is a tissue only few millimeter thick, but with excellent wear characteristics. These properties makes it ideally suited for placement in joints such as the knee and hip. Mechanical and structural capacity of articular cartilage is owed to its unique and complex architecture (Fox et al, 2009; Ergelett et al, 2008; Al-Rubeai & Melero-Martin, 2007).

1.2.1. Structure and composition

1.2.1.1. Composition of articular cartilage

Articular cartilage is not a homogeneous tissue. It has a highly organized structure with biphasic nature, consisting of fluid (mostly water) and a solid, porous-permeable, dense ECM which contributes to its biomechanical function (Cohen et al, 1998). Like other types of cartilage, articular cartilage lacks blood vessels, lymphatic vessels, nerves, inflammatory cells and fibroblasts (Horvai, 2011).

Normal articular cartilage is composed of only single cell type, the chondrocyte. They originate from immature mesenchymal cells that differentiate from somatic or visceral mesoderm during early development and occupy about 2% of the total volume of articular cartilage (Horvai, 2011).

Chondrocytes may appear in the groups of up to eight cells called isogenous aggregates, emerged from the mitotic divisions of a single chondrocyte. As chondrocytes start to produce and secrete ECM components, they are pushed apart from aggregates and become separated each in its own surrounding space (lacuna) (Fig 1) (Mescher, 2013). This microenvironment traps cells within their own matrix which prevents chondrocyte migration or formation of direct cell-to-cell contacts (Fox et al, 2009).
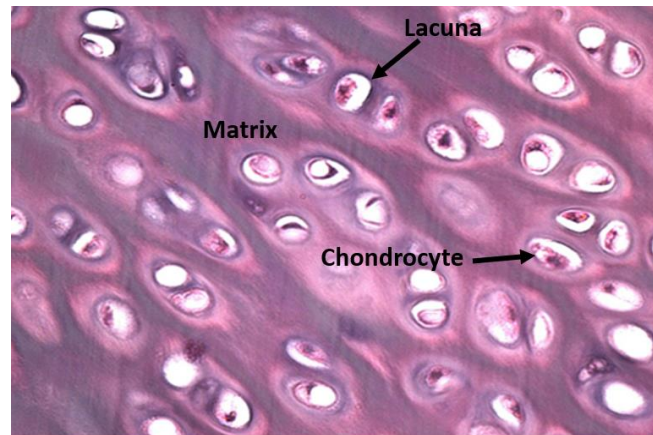
Figure 1. Location of articular chondrocytes. Each cell resides in lacuna surrounded by extensive ECM
(downloaded from: www.studyblue.com).

Depending on the location within cartilage, chondrocytes can exhibit different sizes and shapes. In the deeper zones, they are normally rounded or polygonal, while those at the periphery may be flattened or discoid with the long axis parallel to the surface of the cartilage (Mescher, 2013; Archer & Francis-West, 2003).

Structurally, chondrocytes show intracellular features characteristic for metabolically active cells responsible for production and maintenance of cartilage ECM (Fig. 2) (Fox et al, 2009; Archer & Francis-West, 2003). They produce structural macromolecules essential for tissue formation (collagens and proteoglycans), but also make variety of metalloproteinases (collagenases, gelatinases, aggrecanases) which are responsible for tissue turnover (Roughley, 2002).
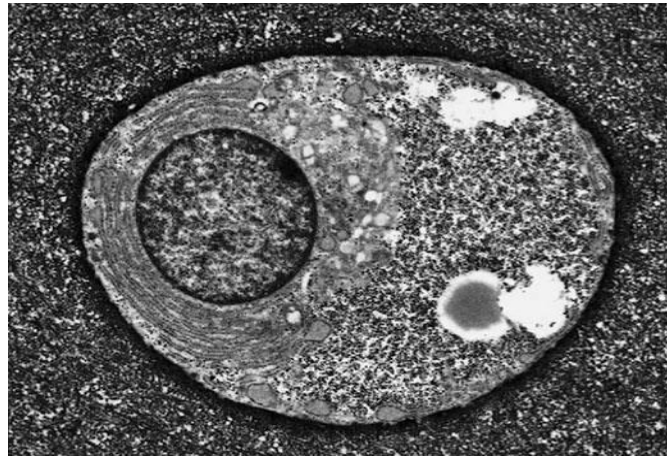
Figure 2. Electron micrograph of articular chondrocyte. The cell is typically rounded with prominent Golgi apparatus and rER which is associated with the production and secretion of cartilage ECM components (adapted from Archer & Francis-West, 2003).

Beside ECM production, chondrocytes have important function in growth, especially that associated epiphyseal plates in the long bones. It is achieved through three basic mechanisms: cell proliferation, matrix secretion and increased cell volume that occurs during hypertrophy (terminal differentiation) (Archer & Francis-West, 2003).

Since articular cartilage is not vascularized, nutrient and metabolite exchange occurs through diffusion from synovial fluid. Therefore, the metabolism of chondrocytes is adapted to low oxygen tension which ranges from 10% at the surface to <1% in the deeper layers. As a result, chondrocytes do not contain abundant mitochondria and produce energy mostly by glycolysis (Bhosale & Richardson, 2008; Archer & Francis-West, 2003).

Cartilage ECM is composed mostly of water, collagen, proteoglycans and other noncollagenous proteins and glycoproteins in lesser amounts. It protects chondrocytes from mechanical damage and has an important role in maintaining chondrocyte phenotype and shape. Components like nutrients, substrates, synthesized and degraded molecules, metabolic waste, cytokines and growth factors are stored or pass through the matrix (Gaut & Sugaya, 2015).

The most abundant component of cartilage ECM is water which makes 80% of cartilage wet weight (Fox et al, 2009). The high water content is owed to proteoglycans which attract water due to their negative charge. It is unevenly distributed throughout cartilage with the highest concentration on the surface. The constant movement and

diffusion of water through cartilage matrix is important for transport and delivery of nutrients to chondrocytes (Horvai, 2011).

Collagen represents 60% of the dry weight of cartilage which makes it the most abundant structural macromolecule in ECM (Fox et al, 2009). Different types of collagen are present in the cartilage and can be divided into fibril-forming and non-fibril-forming collagens (Hu & Athanasiou, 2010). Types II, IX and XI form the core fibrillar network responsible for cartilage's resilience and ability to endure sheer forces (Ergelett et al, 2008; Eyre, 2002). Other types of collagen (I, III, IV, V, VI, X, XII, XIV) do not form fibers, but contribute to ECM structure by enabling formation and stabilizing the type II collagen fibril network. Type II collagen is the most abundant type of collagen in hyaline articular cartilage and represents 90-95% of collagen present in the ECM (Fox et al, 2009). It supports chondrocyte adhesion and induces phenotypic differentiation of cells (Meyer et al, 2006). The entire collagen fibrillar network is stabilized by 100 000 Da protein cartilage oligomeric matrix protein (COMP) (Hu & Athanasiou, 2010).

Proteoglycans are heavily glycosylated proteins consisted of a protein core with covalently attached chains of glycosaminoglycans (GAG). Glycosaminoglycans are long non-branching polysaccharides built of repeating disaccharide units. The presence of sulphate (SO_4^{2-}) and carboxylate (COO^-) ionic groups gives an overall negative charge to GAG macromolecules. Keratan sulfate and chondroitin sulfate are the most abundant types of GAG in articular cartilage (Hu & Athanasiou, 2010). Among proteoglycans the most important are aggrecan, decorin, biglycan and fibromodulin. The most abundant and largest in size is aggrecan (Fox et al, 2009). It is composed of a large core protein with attached side chains of keratan sulfate and chondroitin sulfate. It occupies interfibrillar space of cartilage ECM and aggregates with large, unbranched polysaccharide hyaluronan via link proteins (Fig 3). Negatively charged GAG side chains repel each other which gives aggrecan a shape similar to a bottle brush. The overall negative charge of aggrecan attracts positively charged ions and molecules and creates a positive osmotic pressure which causes a matrix to swell. The swelling force is balanced by collagen fibers. This process gives cartilage its biomechanical properties and allows it to resist compressive loads (Bhosale & Richardson, 2008). Aggrecan synthesis is an important hallmark of chondrogenesis and represents a key marker in research of its molecular mechanisms (Knudson & Knudson, 2001).
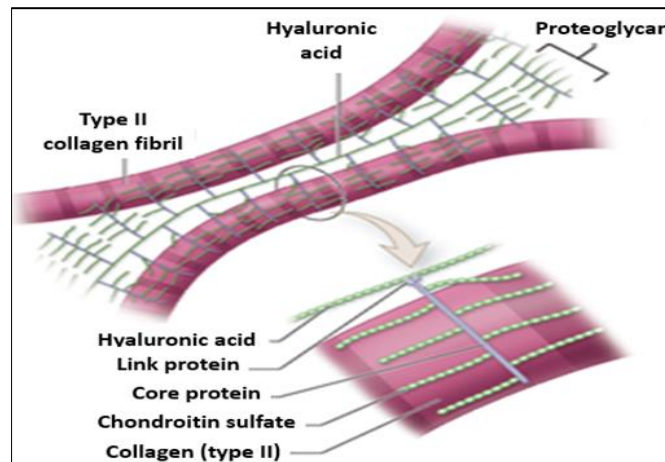
Figure 3. A schematic representation of structure and interaction of key molecular components of cartilage ECM: collagen type II fibrils and proteoglycans linked to hyaluronic acid (adapted from Mescher, 2013)

1.2.1.2. Structure of articular cartilage

Based on the differences in cell shape, size and activity, as well as distribution and arrangement of main ECM components, articular cartilage can be divided in four distinct structural zones: superficial (tangential), middle (transitional), deep and calcified cartilage (Fig 4)(Duarte Campos et al, 2012).

The superficial zone occupies 10-20% of the total thickness of articular cartilage. This zone contains high number of ellipsoid-shaped, flattened chondrocytes with the long axis parallel to the surface. Matrix in this zone is characterized by high collagen concentration (~86% dry weight) and low proteoglycan concentration (~15% dry weight) (Hu & Athanasiou, 2010). Collagen fibrils (mainly composed of collagen type II and IX) are aligned parallel to the surface of cartilage. Fibronectin and water concentration are highest in this zone (Buckwalter et al, 2005). Superficial chondrocytes and synoviocytes produce lubricating components, mainly glycoprotein lubricin and superficial zone protein which form a protective layer on the surface of cartilage. Its main function is reduction of friction and wear (Mollenhauer, 2008; Rhee et al, 2005).

The middle zone makes 40-60% of the total thickness of articular cartilage. This layer contains randomly organized collagen fibrils and lower number of rounded chondrocytes. Water and collagen contents in this zone are lower compared to superficial zone. Proteoglycan content is the highest in this layer (~25% dry weight) (Fox et al, 2009).

The deep zone makes 30% of total thickness of articular cartilage. This layer is characterized by thick, perpendicularly arranged collagen fibrils, high proteoglycan and low water content. These properties give this layer the greatest resistance to compressive forces (Fox et al, 2009). Tidemark is a calcified region that separates deep from calcified zone.

The calcified zone serves as a transition between articular cartilage and underlying subchondral bone. It contains minimal amounts of collagen and water. Chondrocytes are smaller in volume and embedded in a mineralized matrix. They show minimal metabolic activity and express hypertrophic phenotype characterized by synthesis of collagen type X (Bhosale & Richardson, 2008).
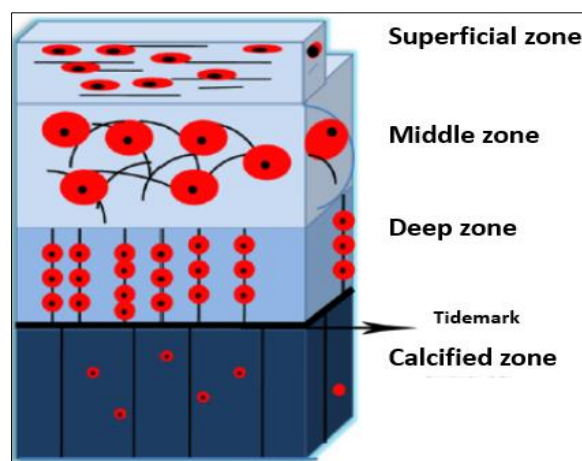
Figure 4. Zonal organization of articular cartilage. Zones differ in number and shape of chondrocytes as well as the organization, density and thickness of collagen fibrils (from Doulabi et al, 2014)

1.2.2. Damage and repair

Articular cartilage damage can be the result of degenerative joint diseases, most commonly osteoarthritis, different genetic or metabolic conditions and trauma (Hunziker, 2001). Articular cartilage lesions generally do not heal or heal only partially under certain biologic conditions. It has been known for a long time that this tissue has weak or no capacity to repair itself. Even in 1743 famous Scottish anatomist and physician William Hunter reported: "Ulcerated cartilage is a troublesome thing...once destroyed it is not repaired." (Chiang & Jiang, 2009). The poor ability of articular cartilage for spontaneous self-repair is the result of low cellularity of the tissue, immobility of chondrocytes, limited

ability of mature chondrocytes for proliferation, avascularity of the tissue and, consequently, inaccessibility of undifferentiated progenitor cells that can promote repair (Temenoff & Mikos, 2001; Ghivizzani et al, 2000).

The manner in which articular cartilage responds to the traumatic injury depends on the type of the injury. Traumatic injuries fall into three categories: microdamage, chondral lesions and osteochondral lesions (Frenkel & Di Cesare, 1999). Microdamage is a type of injury that includes loss of proteoglycans or other ECM components and is not visible on the articular surface. Chondrocytes recognize these changes and respond by increased production of ECM components. As long as produced ECM is greater or equal to the amount of lost ECM, homeostasis of tissue is preserved and tissue can recover. Otherwise, chondrocytes are no longer protected by matrix and tissue degenerates. The result of these focal mechanical disruptions are chondral injuries like chondral fissures and flaps (Hu & Athanasiou, 2010). However, because chondrocytes are unable to produce large amounts of ECM, in the case of accumulated microdamage, originally small focal defects usually progress and finally result in destruction of the entire thickness of articular cartilage layer. These so called chondral lesions do not heal spontaneously.

On the other hand, osteochondral or full thickness defects which penetrate to subchondral bone and bone marrow spaces do have a potential for intrinsic repair (Getgood et al, 2009). This kind of defect causes lesions of bone blood vessels. Soon after injury, blood from damaged blood vessels forms a hematoma which fills the injury site. Fibrin forms inside the blood clot and platelets bind to collagen fibrillar network. Platelets release many bioactive factors including platelet-derived growth factor (PDGF) and transforming growth factor beta (TGF-β) (Buckwalter, 1998). Beside platelets, large quantities of growth factors are also released from the bone like TGF-β, PDGF, bone morphogenic proteins (BMPs), insulin like growth factors I and II (IGF-I,-II). These growth factors play a key role in initiation of repair of osteochondral defects by stimulating vascular invasion and migration of stem cells on the site of injury. Different kinds of stem cells are involved in the repair process originating from bone marrow spaces, adipose tissue, vascular and perivascular tissue, bone and synovium (Buckwalter, 1998; Hunziker, 2001). Recruited stem cells differentiate in chondrocyte-like cells which produce matrix of proteoglycans, collagen type II and I. However, matrix degenerates and formed tissue is gradually replaced with more fibrous tissue. Also a shift in collagen production occurs, from collagen type II to predominantly type I (Frenkel & Di Cesare, 1999). At the end of

repair process, formed tissue resembles a mixture of hyaline cartilage and fibrocartilage and shows different characteristics compared to native articular cartilage. It lacks structural organization, contains higher amount of collagen type I, shows inferior biomechanical properties and usually degrades with time (Steinert et al, 2007; Redman et al, 2005). For these reasons, osteochondral lesions rarely heal completely and often lead to occurrence of osteoarthritis.

Except for the depth of cartilage damage, other important factor in the repair response is the size of the defect. Study in horses has showed that only those defects smaller than 3 mm in diameter can repair completely after 9 months (Convery et al, 1972; Hurtig et al, 1988). Other important factors are also age, obesity and practicing certain kind of sport where is higher probability for traumatic injuries (Bhosale & Richardson, 2008; Mithoefer et al, 2008).

The most common degenerative disease of synovial joints affecting millions of people is osteoarthritis (OA). It is characterized by progressive degeneration of articular cartilage, remodeling of subchondral bone, formation of bone outgrowths or osteophytes and synovitis (Lories & Luiten, 2012). These changes can lead to symptoms such as pain, stiffnes and loss of joint function. It usually affects joints of hand, knee, foot and hip (Wieland et al, 2005). Osteoarthritis is considered to be a multifactorial disease to which both genetic and acquired factors contribute. Ageing increases the risk for OA by compromising the balance between anabolic and catabolic processes in the cartilage tissue (Martin & Buckwalter, 2002). Obesity and history of joint trauma are also proven to be important risk factors for OA (Wieland et al 2005). In some families with cases of OA, an inheritance of mutant allele for type II procollagen gene (COL2A1) has been identified (Frenkel & Di Cesare, 1999).

1.2.3. Treatment options

Highly organized structure and limited repair capacity of articular cartilage make it extremely challenging to treat cartilage injuries medically. For the past three centuries physicians and scientists have tried to find effective therapies and procedures in order to accomplish repair or regeneration of articular cartilage after degeneration or traumatic damage. These methods fall into three categories or approaches (Steinert et al, 2007).

The first approach is based on mechanical penetration of subchondral bone by abrasion or drilling. It causes bleeding and induces natural repair response through stem cell migration and differentiation. These techniques include microfracture, abrasion arthroplasty and Pridie drilling (Redman et al, 2005; Bhosale & Richardson, 2008). Microfracture is cheap, easy to perform, minimally invasive and thus mostly used technique. However, the final outcome is the formation of fibrocartilagenous tissue with different biomechanical properties (Getgood et al, 2009).

The second approach includes transplantation of pieces of tissue in the place of a full thickness cartilage defect. Damage site can be filled with small plugs from a less weight bearing region of the joint (autografts) and the method is called mosaicplasty, or with a full allograft mostly derived from cadaveric donors (Redman et al, 2005). The main limitation of mosaicplasty is a small amount of cartilage in the body available for transplantation to other sites. Important issues that need to be questioned are the effects of damage to the donor site as well as the ability of autograft from a less weight bearing region to withstand the forces at the joint surface (Temenoff & Mikos, 2001). Allograft transplantations proved to be a successful treatment, with 95% survival rate at five years (O'Driscoll, 1998). However, there have been cases of immunologic response to fresh allografts after transplantation. Beside osteochondral grafts, perichondrium and periosteum grafts are also used (Redman et al, 2005).

The third approach is based on transplantation of cells. Since it has been performed for the first time in 1987, autologous chondrocyte transplantation (ACT) or autologous chondrocyte implantation (ACI) has proved to be promising. The entire procedure of ACI in humans was firstly described by Brittberg et al (1994). The procedure involves isolation of patient's own chondrocytes from small slices of cartilage. The extracellular matrix is removed by enzymatic digestion and cells are then expanded in a monolayer culture. Perisoteal flap is sutured over the defect and cultured autologous chondrocytes are injected under the flap. An improved and modified version of this method is matrix-induced autologous chondrocyte implantation (MACI) where chondrocytes are combined with matrix made of resorbable biomaterials. Biomaterials enhance proliferation and differentiation of cells (Fig. 5) (Marlovits et al, 2006).
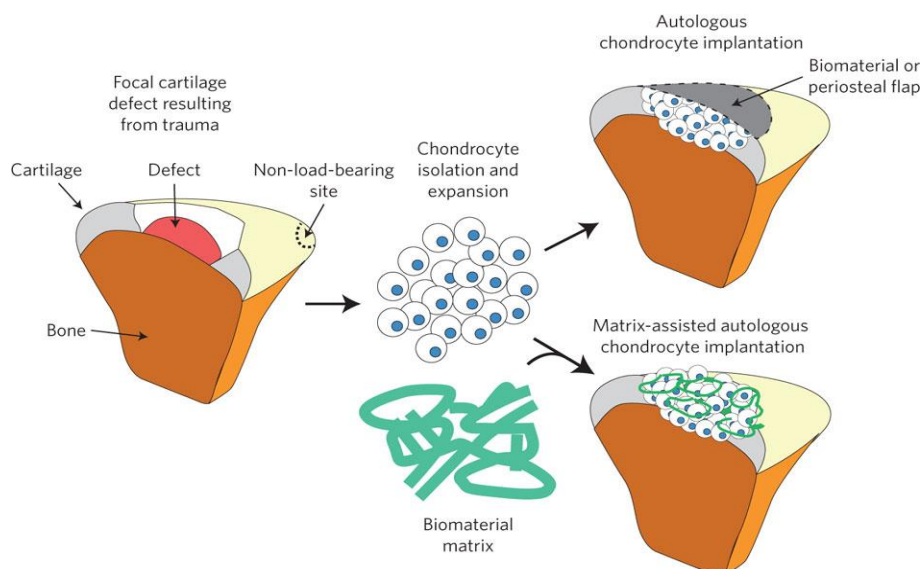
Figure 5. Basic principles and comparison of autologous chondrocyte implantation (ACI) and matrix-induced chondrocyte implantation (MACI) methods
(from Ducheyne et al, 2012)

1.3. Cartilage tissue engineering

Despite the availability of various cartilage defect treatment options, none of them proved to be entirely effective in restoration or regeneration of hyaline cartilage tissue. The final outcome is mostly the formation of fibrocartilagenous tissue characterized by higher amount of collagen type I, different structure and biomechanical properties inferior to native articular cartilage. Growing problems of obesity and ageing of overall population will lead to dramatic increase in cases of osteoarthritis and other cartilage pathologies in general. Therefore, cartilage diseases and defects are becoming a major socioeconomic and medical problem (Chung & Burdick, 2008).

Tissue engineering (TE) is an evolving field that has a potential to revolutionize medicine and provide permanent solutions to tissue damage and tissue loss. The goal of TE is to provide living biological/physiological substitutes that could replace tissue loss due to diseases, congenital abnormalities or trauma. This substitute should be able to perform similar biological functions and have similar structural and morphological features as native tissue (Nesic et al, 2006; Hardingham, 2002). Tissue engineering is a multidisciplinary field that combines knowledge from cell and molecular biology, material science, engineering and medicine (Vunjak-Novakovic, 2006). It is based on manipulation of several key components and their combinations: i) cells which produce desired tissue, ii) scaffolds which promote proliferation and differentiation of cells by providing a three-

dimensional environment, iii) biochemical and physical factors which provide signals that direct cell's development towards a certain phenotype (Gomes & Reis, 2004). To date, great advances have been achieved in the field of TE of different tissues and organs (Atala, 2009; Amiel et al, 2006; De Filippo et al, 2004; Vesely, 2005; Kulig & Vacanti, 2004).

Cartilage TE is not an entirely new approach. Above mentioned methods ACI and MACI can be considered as forerunners of cartilage TE. The main goal of cartilage TE is to produce cartilage tissue with similar structure and properties *ex vivo* which can be then implanted on the site of the defect in the joint. The implant should be able to provide natural repair and with time become integrated with the patient's tissue (Hardingham, 2002). However, to date, the properties and structure of native articular cartilage have not been entirely mimicked by TE methods. Therefore the main challenge of cartilage TE is to determine a right combination of appropriate cell type and matrix scaffold together with biochemical and physical factors which will ultimately result in articular cartilage formation. This requires intensive research that deepen our understanding of natural process of chondrogenesis and articular cartilage biology. These findings enable constant improvements and optimization of cartilage TE components.

1.3.1. Cell source

One of the basic challenges of cartilage TE is to identify an ideal cell source. It should be easy to isolate, capable of expansion and production of cartilage-specific ECM molecules (collagen type II and aggrecan) (Chung & Burdick, 2008). Chondrocytes are the most obvious choice since they are found in natural cartilage and have been extensively researched for cartilage repair (Kisiday et al, 2002). Other potential source are fibroblasts which can be easily isolated, expanded and directed toward chondrogenic phenotype (Nicoll et al, 2001). Recently, mesenchymal stem cells have been considered as potential cell source for cartilage TE. These cells can be isolated from many different tissues and show great capacity for differentiation *in vitro*. Additionally, all these cell types can be modified genetically to induce or enhance chondrogenesis (Madry et al, 2005).

1.3.1.1. Chondrocytes

Chondrocytes have been widely used for the purposes of cartilage TE. Adult chondrocytes have been isolated from various sources like articular cartilage, nasal septum, ribs or ear cartilage and used for TE (Bhardwaj et al, 2015). Since they comprise only a small percentage of articular cartilage, prior to use a necessary step is their expansion in monolayer cultures. However, such culture causes these cells to lose their specific phenotype and this phenomenon is described in the literature as chondrocyte dedifferentiation (Freyria & Mallein-Gerin, 2012). This process is characterized by decreased synthesis of proteoglycans and collagen type II expression, increased expression of collagen type I and change to more fibroblast-like phenotype. Changes in expression of several other genes also accompany this process (integrins, growth factors, matrix modulators, different kinases) (Chung & Burdick, 2008). Darling et al (2005) showed changes in articular chondrocyte gene expression as early as the first passage. The use of passaged, dedifferentiated chondrocytes for cartilage TE can lead to compromised tissue quality with decreased biomechanical properties. Three-dimensional cultures like alginate, agarose beads and fibrin glue may preserve chondrocyte phenotype (Bhardwaj et al, 2015). Additionally, a variety of methods have been employed to redifferentiate passaged chondrocytes: 3D scaffolds, bioreactors, reduced oxygen tension and addition of different growth factors (TGFβ, fibroblast growth factor (FGF) and IGF) (Chung & Burdick, 2008).

1.3.1.2. Mesenchymal stem cells

Mesenchymal stem cells (MSCs) are self-renewing pluripotent cells that have a potential to differentiate into chondrocytes, adipocytes, osteoblasts, fibroblasts and other tissues of mesenchymal origin (Fig 6). They reside in many tissues within the adult organism. Multipotency and great proliferation capacity make them a valuable cell source for TE in general (Bhardwaj et al, 2015). The chondrogenic potential of stem cells from various tissues has been investigated (muscle, periosteum, synovial membrane, adipose tissue), but those originating from bone marrow seems to be the most used for cartilage TE (Freyria & Mallein-Gerin, 2012). Mesenchymal stem cells are rare in bone marrow,

representing approximately 1 in 10,000 nucleated cells (Chamberlain et al, 2007). For their application in cartilage TE, it is necessary to amplify them *in vitro* to obtain sufficient cell number for laboratory and clinical purposes. Afterwards, the expanded bone marrow stem cells (BMSCs) need to be exposed to specific chemical, physical and/or mechanical factors that will promote chondrogenic differentiation (Freyria & Mallein-Gerin, 2012). Crucial factors and conditions that serve as chondrogenic signals and promote chondrogenesis are discussed in the chapter 1.3.3.
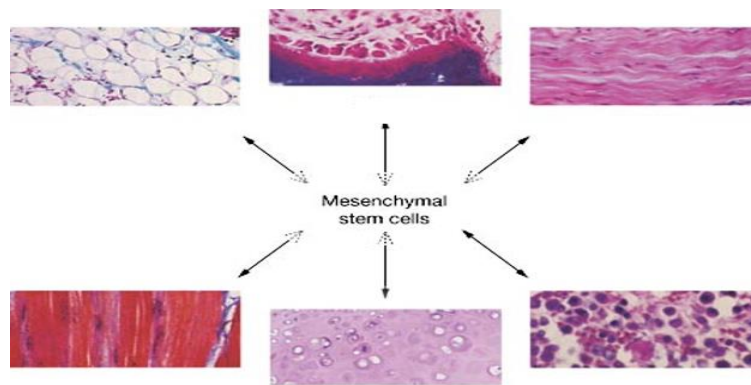
Figure 6. The multilineage differentiation potential of mesenchymal stem cells (MSCs).
Under appropriate conditions MSCs can differentiate into different tissues: adipose tissue, bone, tendon (upper row, from left to right); muscle, cartilage, stroma (lower row, from left to right) (from Chen & Tuan, 2008).

1.3.2. Scaffolds

Scaffolds represent one of the key components of TE approach. Scaffold materials facilitate the attachment, proliferation, differentiation of embedded cells and function as a template which controls the geometry of newly formed tissue (Moutos & Guilak, 2008). The choice of scaffold material depends on the type of tissue that is being engineered, *i.e.* different tissues have different requirements for success, which has led to development of various materials with unique characteristics. However, each scaffold that is being used needs to be biocompatible, provoking no inflammation or immune reaction; biodegradable, to enable newly formed tissue to replace the scaffold; porous, to enable cell migration and nutrient exchange and biomimetic, to mimic structure and function of native tissue (Smith & Grande, 2015).

To date, a wide range of natural and synthetic materials have been investigated as scaffolds in cartilage TE. Natural polymers that have been explored as bioactive scaffolds

for cartilage TE include collagen, silk, fibrin, alginate, agarose, gelatin, chitosan, hyaluronic acid (HA) and cellulose (Bhardwaj et al, 2015). Natural polymers are similar to ECM and often interact with cells via surface receptors and affect cell function. However, due to this interaction, they can stimulate an immune system response. Additionally, they may be inferior mechanically and subject to enzymatic host degradation (Chung & Burdick, 2008). On the other hand, chemistry and properties of synthetic polymers are controllable and reproducible. Different chemical and physical parameters of a synthetic polymer can be modified to alter their mechanical and degradation characteristics (Drury & Mooney, 2003). Synthetic polymers that are currently being explored for application in cartilage TE include poly (glycolic acid) (PGA), poly (lactic) acid (PLA), poly (ethylene-glycol) (PEG), polyurethanes, etc. (Bhardwaj et al, 2015). Drawbacks to synthetic scaffolds include risk of rejection and inflammatory response as well as forming of degradation byproducts that may be toxic and negatively influence on cells and surrounding tissue (Gaut & Sugaya, 2015).

Since recently, composite scaffolds have been developed in order to overcome certain disadvantages of single-phase biomaterials. They are mostly composed of a mixture of natural and synthetic polymers and allow the creation of more complex geometries and better functional properties more representative to articular cartilage (Gaut & Sugaya, 2015).

1.3.2.1. Peptide hydrogel scaffold

Hydrogels are materials consisted of a water-swollen, three-dimensional (3D) network which permits attachment of molecules and cells. These scaffolds serve as a substitute for natural ECM to organize cells into 3D architecture and to present stimuli which direct the growth and a formation of a desired tissue. Currently, they are being widely used for the purposes of TE (Seow & Hauser, 2014).

Hydrogels can be consisted of natural or synthetic materials which are able to form a gel after physical, ionic or covalent crosslinking (Liu et al, 2010). Protein-based scaffolds are biodegradable materials with high water content. They provide temporary support for cell growth and maintenance as they facilitate the exchange of nutrients, gases, metabolic waste and cell signaling molecules (Amini & Nair, 2012).

In the early 1990s ionic self-complimentary self-assembling peptides were discovered (Koutsopoulos, 2009). These peptides consist of alternating hydrophilic and hydrophobic amino acids. When dissolved in deionized water, self-assembling peptides form stable β-sheet structures. Exposure to electrolyte solution initiates β-sheet assembly into nanofibers with diameters of 10-20 nm. These nanofibers further organize to form highly hydrated hydrogels (Kisiday et al, 2002; Spiller et al, 2011). Peptide hydrogels do not use harmful materials to initiate solution-gel transformation (eg. toxic cross-linkers) and degradation products are amino acids which can be metabolized. They are biodegradable, non-immunogenic and injectable which allows their application in a minimally invasive manner. Additionally, peptide sequences can be designed for specific cell-matrix interactions that influence cell differentiation and tissue formation (Kisiday et al, 2002). Member of this family is the peptide RADA 16 with a repeating sequence [Ac-(RADA)$_4$-COHN$_2$] or acetyl-(Arg-Ala-Asp-Ala)$_4$)-CONH$_2$ that facilitates hydrogel formation with >99% water content in the presence of salts (Koutsopoulos, 2009). This peptide contains a RAD motif, similar to integrin receptor binding site RGD. It is not known if the RAD repeats behave in the same manner to RGD motifs. Their properties as cell adhesion molecules are being investigated in different cell types (Bokhari et al, 2005). Different cell types embedded in peptide hydrogel RADA as a 3D scaffold showed successful differentiation, migration and production of their own ECM. There are reports of RADA application in cartilage, bone and brain TE (Kisiday et al, 2002; Spiller et al, 2011; Cheng et al, 2013). Another important potential application of peptide hydrogels is for controlled release of small molecules which can be entrapped within. Peptide hydrogels are biocompatible which means that they slowly degrade and release entrapped molecules. Controlled release of signal molecules like growth factors or drugs can be useful in TE for controlled delivery of inductive signals that promote differentiation and tissue formation (Koutsopoulos, 2009).

1.3.3. Chondrogenic signals

Third component of tissue engineering are different stimulating factors able to induce, accelerate and enhance cartilage tissue formation. First group are different growth factors and other additives that affect cellular differentiation and cartilage tissue formation. Growth factors like TGF-β, FGF, BMP and IGF together with other soluble

factors like insulin have been explored for their effects on cartilage TE. Among these molecules, TGF-β superfamily of cytokines play a major role in cartilage tissue formation (Chung & Burdick, 2008). The TGF-β superfamily is comprised of more than forty members, including the BMPs. They are involved in the processes of cell proliferation, differentiation, migration and survival (Gordon & Blobe, 2008). TGF-β1, 2 and 3 induce chondrogenesis and stimulate synthesis of ECM in both chondrocytes and MSCs. They trigger the signaling cascade that activates expression of *SOX9*, a key transcription factor necessary for early chondrogenesis. *SOX9* activates promoters of genes coding for collagen type II, aggrecan and other cartilage-building proteins (Fig 7) (Gaut & Sugaya, 2015). BMP-2 and -7 together with IGF-1 also promote ECM synthesis. FGF-2 preserves chondrogenic potential of monolayer expanded chondrocytes and enhances proliferation (Chung & Burdick, 2008).

In addition to growth factors, many protocols for chondrogenic differentiation also include ascorbic acid and dexamethasone. Ascorbic acid is required for collagen and proteoglycan synthesis. It is also shown that stimulates differentiation and proliferation of MSCs (Choi et al, 2008). Dexamethasone (DEX) is a synthetic glucocorticoid that showed ability to direct regulation of cartilage-specific genes in culture of MSCs. Glucocorticoids usually act as inhibitors of chondrocyte cell growth and ECM synthesis, but reports are suggesting that specific DEX concentrations seem to have synergistic effect with chondrogenic growth factors (especially TGF-β1) and thus contribute to chondrogenesis of MSCs (Awad et al, 2003).

In natural environment *i.e.* in the joint, chondrocytes are exposed to reduced oxygen tension (6-10% at the surface and only 2% in the deep zone) and elevated hydrostatic pressure. Mimicking of these conditions represents a new additional strategy for improvement of *in vitro* cartilage formation (Chung & Burdick, 2008). Studies suggest that culturing of MSCs in hypoxic conditions (5% O_2) promotes expression of chondrogenic transcription factors, including SOX transcription factors, synthesis of ECM and collagen type II production (Gaut & Sugaya, 2015). The transcription factor, hypoxia-inducible factor-Iα (HIF-Iα) is a crucial mediator of this cellular response to hypoxia (Fig 7) (Kanichai et al, 2008). Chondrocytes in the joint are also exposed to mechanical loading and forces which affects their metabolism and enables nutrient and oxygen exchange. Several studies confirmed that application of mechanical stimulation *in vitro* promotes differentiation and matrix production. A significant challenge remains to determine the

optimal frequency, type and timing of mechanical loading on cultures for cartilage TE (Gaut & Sugaya, 2015; Laganà et al, 2008; Vunjak-Novakovic et al, 2002).
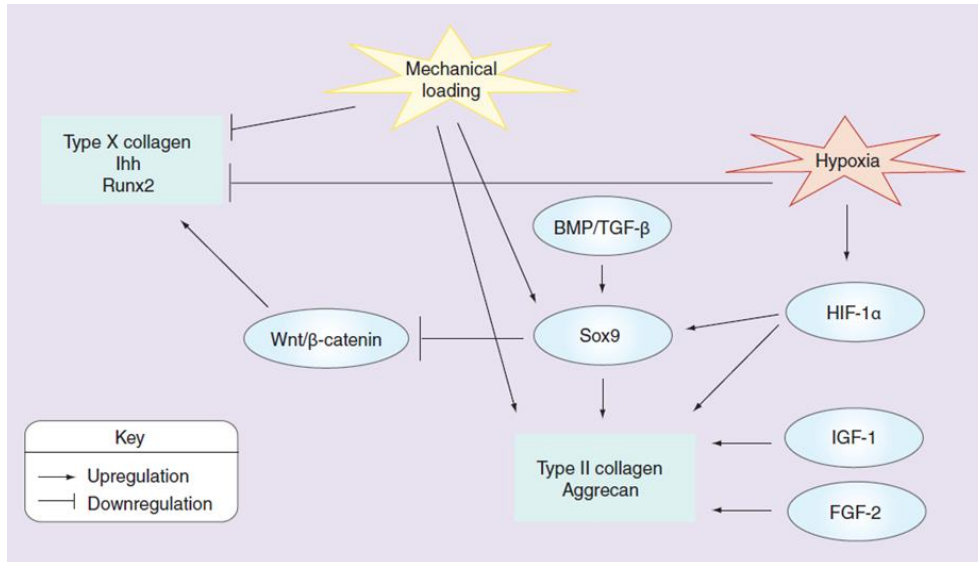
Figure 7. Basic stimuli and signaling pathways modulating mRNA expression of chondrogenic genes. Growth factors, hypoxic conditions and mechanical stimulation promote production of cartilage-specific proteins while inhibiting pathways that lead to hypertrophy and osteogenesis (from Gaut & Sugaya, 2015).

2. OBJECTIVE OF THE STUDY

The primary goal of TE approach is to produce tissue implants that structurally and functionally resemble native tissue that needs to be repaired. For successful engineering of each tissue, a right combination of cell type, scaffold and inductive signals (chemical and physical) must be used. Poor ability of self-repair, ineffectiveness of common treatment options together with the growing problems of obesity and ageing of general population, make the articular cartilage an ideal candidate for TE approach. Again, the ultimate challenge is to choose the right combination of main TE components in order to produce functional cartilage tissue. This research is an attempt of optimization of conditions for cartilage tissue engineering. The specific goals of this research are:

- To establish a 3D culture system for cell growth in a self-assembling peptide hydrogel RADA
- To determine and compare chondrogenic potential of three different cell types: chondrocytes, human bone marrow stem cells (hBMSCc) and a combination of chondrocytes and hBMSCs in 2:1 ratio
- To demonstrate the effect of oxygen tension on the efficiency of chondrogenic differentiation
- To demonstrate the influence of dexamethasone source (in chondrogenic medium or incorporated within the peptide hydrogel scaffold) on chondrogenesis
- To determine the kinetics of dexamethasone release from the peptide hydrogel scaffold

Hypothesis: chondrocytes cultivated in a co-culture with hBMSCs will improve their proliferation and chondrogenesis. Hypoxic conditions will enhance efficiency of chondrogenesis since oxygen tension in cartilage is naturally low. Dexamethasone source will not influence on efficiency of chondrogenesis, but addition of dexamethasone in the hydrogel scaffold will make cultivation easier since it does not need to be added in the medium.

3. MATERIALS AND METHODS

3.1. Isolation of human articular chondrocytes

Chondrocytes were isolated from articular cartilage of the knee obtained after knee surgery performed at clinical hospital „Sveti Duh" in Zagreb with approval of the patient and Ethical comitee. Samples of cartilage tissue were put in the physiological solution and kept on ice until isolation. Isolation was performed soon after in the sterile conditions. Samples were put in PBS and sliced with sharp sterile scalpel blade as finely as possible. Cutting of the sample in the small pieces improves subsequent enzymatic digestion of the tissue. Pieces of tissue and PBS were then centifuged for 5 min at 300 g and 4 °C. Supernatant was removed and 0,08% bacterial colagenase II in DMEM (Dulbecco's Modified Eagle's Medium, Lonza) was added. Samples were incubated in DMEM/colagenase over night at 37 °C. Once digested, the cellular suspension was strained through commercially available cell strainers (BD Falcon, 100 μm pore size) to remove large particles and centrifuged at 300 g for 5 min to obtain a cell pellet. Cells were resuspended in high-glucose DMEM supplemented with 10% FBS (Gibco), 1% penicillin/streptomycin (Lonza) and 1% L-glutamine (100 x GlutaMAX, Gibco) and plated into Petri dishes at low cell density. Cells were grown at 37°C and 5% CO_2 with media changes every 3 days. When confluence was reached, cells were rinsed with PBS and trypsinized with 2 mL of trypsin (0,25% trypsin, Sigma) per each Petri dish to promote detachment of cells. After 5 min, trypsin was deactivated with addition of growth medium and cell suspension was centrifuged at 300 g for 5 min. Pellet was resuspended in freeze medium containing 80% high-glucose DMEM, 10% DMSO (Sigma) and 10% FBS. Suspension was transferred in 2 mL cryo-vials, freezed at -80°C and than transferred in liquid nitrogen until use.

3.2. Expansion of human articular chondrocytes

Tube containing chondrocytes in freeze medium was removed from liquid nitrogen and thawed in a 37 °C water bath. Thawing is a stressful process for cells and needs to be performed as quickly as possible to ensure viability and functionality of cells.

After thawing, cell suspension was transferred in high-glucose DMEM (containing 10% FBS, 1% penicillin/streptomycin and 1% L-glutamine) and centrifuged for 7 min at 300 g and 4 ℃ in order to remove DMSO. Supernatant was removed while cells were resuspended in 10 mL of DMEM per large Petri dish and put in incubator at 37 ℃, 5% CO_2. After 24 h chondrocytes were observed under inverted light microscope. They were attached to the plastic surface of the Petri dish and medium was changed. Confluence was reached 3 days after and chondrocytes needed to be transferred to another Petri dish in a lower cell density in order to keep their ability of dividing and growth. Each transfer of cells to the new dishes in a lower cell density is called a passage. Cells were passaged until sufficient number of cells was produced. Cells were washed with PBS, trypsinized with 2 mL of trypsin per each dish and put in an incubator. After 5 min, trypsin was inactivated with addition of 4 mL of DMEM. Cells from each Petri dish were transferred in two new dishes. Medium was added up to a volume of 10 mL per dish. Medium change was performed 3 days after. Therefore, chondrocytes were passaged only once.

3.3. Expansion of human bone marrow mesenchymal stem cells (hBMSCs)

Tube with human bone marrow derived mesenchymal stem cells (hBMSCs) was removed from liquid nitrogen and thawed in water bath. Cells were put in medium and centrifuged for 7 min at 300 g and 4℃ to remove DMSO. Pellet was resuspended in 4 mL of low-glucose DMEM (Lonza) containing 10% FBS, 1% L-glutamine, 1% penicillin/streptomycin with addition of a growth factor FGF-2 (Gibco) in a concentration of 10 ng/mL. This medium keeps hBMSCs in an undifferentiated state and contributes to their proliferation. Cell suspension was transferred in cell culture flask (BD Falcon, 250 mL, polystyrene) and filled with medium up to 14 mL. Cells were put in incubator at 37 ℃, 5% CO_2. After 24 h mesenchymal stem cells were attached, while certain amount of hematopoietic stem cells that were isolated from bone marrow together with mesenchymal stem cells remained floating in the medium. Media exchange thus removes hematopoietic and leaves only mesenchymal stem cells. Cells were washed with PBS and 14 mL of new media was added. Cells were passaged 2 times with media exchange every 2 days.

3.4. Chondrogenic differentiation

3.4.1. Differentiation medium

Chondrogenic medium was prepared according to protocol developed by Center for Advanced Orthopaedic Studies, Beth Israel Deaconess Medical Center, Harvard Medical School, optimized for chondrogenesis of hBMSCs in pellet culture.
Medium was composed of high-glucose DMEM supplemented with 10% FBS and 1% penicillin/strepromycin with addition of following components:

- L-proline in a final concentration of 40 μg/mL (Sigma)
- Ascorbic acid-2-phosphate in a final concentration of 50 μg/mL (Santa Cruz)
- 1 x Insulin-transfferin-selenium (ITS) (Sigma)
- TGFβ-1 in a final concentration of 10 ng/mL (Abcam)

Two different media were prepared. One contained L-proline, ascorbic acid-2-phosphate, ITS and TGFβ-1 and in the second one, beside these components dexamethasone was added in a final concentration of 10^{-7} M (Sigma, diluted in 100% ethanol).

3.4.2. Preparation of cells

One flask with hBMSC containing approximately 10^6 cells was harvested. Cells were washed with PBS and trypsinized with addition of 2 mL of trypsin and put in an incubator at 37 ℃ for 5 min. Trypsin was inactivated with addition of 10 mL of DMEM. Cells were centrifuged for 5 min at 300 g and 4 ℃. Cell pellet is resuspended in 10% sucrose and centrifuged again for 5 min at 300g and 4 ℃. Cell pellet is resuspended in 1 mL of 10% sucrose to obtain a cellular suspension with a concentration of 10^6 cells/mL. Cellular suspension was put on ice until mixing with peptide hydrogel scaffold.

Two large Petri dishes with chondrocytes containing approximately 10^6 cells each were harvested. Cells were washed with PBS, trypsinized with 2 mL of trypsin and put in an incubator for 5 min. In each dish 4 mL of DMEM were added for trypsin inactivation.

Content of both dishes was transferred in the same centrifuge tube (ISOLAB, 15 mL) and centrifuged for 5 min at 300 g and 4℃. Cell pellet was resuspended in 10% sucrose and centrifuged again. Cell pellet was resuspended in 2 mL of 10% sucrose to achieve a final concentration of 10^6 cells/mL. Cells were put on ice until required.

Suspension containing a combination of chondrocytes and hBMSCs in 2:1 ratio was prepared as a mixture of corresponding volumes of previously prepared suspensions of chondrocytes and hBMSCs in a 10% sucrose. Minimal required volume of this suspension is estimated based on the number of cell inserts which should contain combination of chondrocytes and hBMSCs. Suspension is put on ice until mixing with peptide hydrogel.

3.4.3. Peptide hydrogel scaffold with and without dexamethasone

Commercially available Corning®PuraMatrix™ Peptide Hydrogel is used as a scaffold (Fig 8). It is a synthetic matrix, used to create three dimensional microenvironments for a variety of cell culture experiments. It is a member of RADA 16 family, consisted of 1% of standard amino acids and 99% of water. Under physiological conditions peptides self-assemble in a 3D hydrogel (Fig 8).
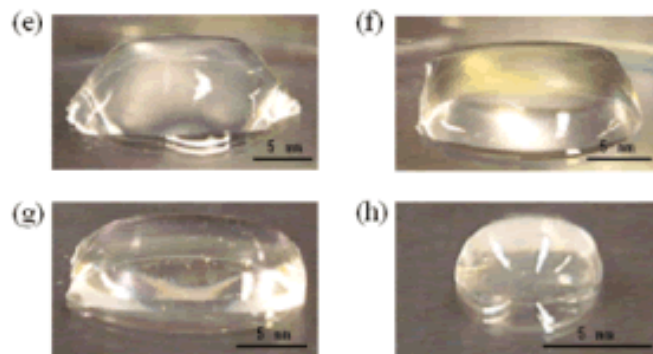
Figure 8. Gelation process of PuraMatrix™ peptide hydrogel. When PuraMatrix™ Peptide Hydrogel is exposed to physiological salt conditions, the peptide self-assembles into hydrogel (from e to h) (from: www.linevikingsson.blogg.se).

Prior to use, a 500 μL aliquot of RADA was vortexed and then mixed with 500 μl of 20% sucrose to generate 2x concentration of RADA in 10% sucrose (addition of sucrose to RADA hydrogel and previously to cells is necessary in order to protect cells until the pH

is normalized by equilibration with tissue culture media during 3D culture establishment).

Two types of peptide hydrogel scaffolds were prepared: one without dexamethasone and other with dexamethasone. Hydrogel without dexamethasone is prepared by aliqoting 50 μL of RADA/sucrose mixture in 1,5 mL tubes. Hydrogel scaffolds with dexamethasone were prepared in 1,5 mL tubes by mixing of 48,5 μl RADA/sucrose suspension with 1,5 μl of dexamethasone (Sigma, 10^{-3} M stock solution). The final concentration of dexamethasone in hydrogel scaffold was 1,5 x 10^{-5} M.

3.4.4. Establishment of three-dimensional cell culture

Three dimensional stationary cell culture was established by encapsulation of cells in the peptide hydrogel placed in cell culture inserts (Falcon 0,4 μm pore size PET track-etched membrane 24 well format cell culture inserts) (Fig 9) inserted in a corresponding 24 well plate. Two such plates were needed, one for growth in normoxic (20% O_2) and other in hypoxic conditions (5% O_2).

Figure 9. Cell culture inserts with a corresponding 24 well plate. Pores at the bottom membrane of cell inserts allow medium flow while flanges on the insert rim facilitate handling (downloaded from www.biolab.com.).

Each plate contained two groups of samples: in one group dexamethasone was added only in the chondrogenic medium and in the other group it was embedded in a hydrogel scaffold and thus was not added in the medium. Each sample group involved

duplicates of hBMSCs, chondrocytes and combination of chondrocytes and hBMSCs in 2:1 ratio (Fig 10).
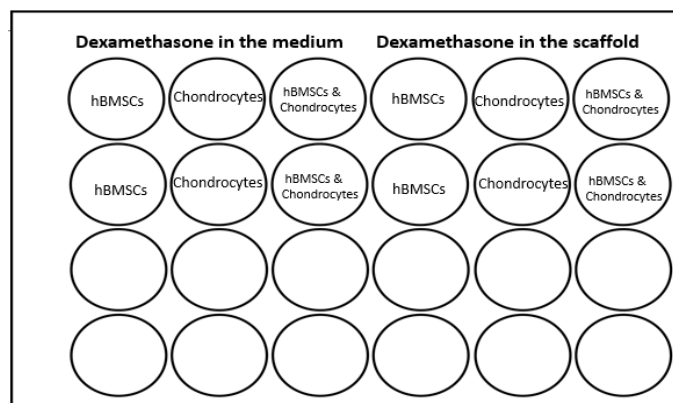
Figure 10. Scheme of types and arrangement of samples on 24 well plates.

Each plate is prepared according to following protocol: desired number of cell culture inserts was placed in wells. Wells were filled by carefull pipetting of 200 μL of corresponding chondrogenic medium down the side of the well, between the insert and the well. Inserts were filled with a mixture of previously prepared cells and RADA in sucrose. The final volume of RADA/sucrose and cell/sucrose mixture in each insert was 100 μL. This mixture is prepared by quick, but gentle pippeting 50 μL of corresponding cell/10% sucrose suspension in 50 μL of corresponding RADA/20% sucrose preparation. Therefore, the final concentration of RADA was 0,25%. Upon preparing, each RADA/cell mixture is gently resuspended and quickly pipetted into the center of the insert. In the moment when all inserts were filled with RADA/cell mixture, in each insert 2 x 200 μL of corresponding medium was added. It was important to work as quickly as possible to minimize the amount of time that cells were in contact with RADA prior to addition of media since RADA exhibits low pH. Salts from the medium triggered self-assembly of 3D hydrogel. To complete the gelation of hydrogel, both plates were incubated for 1 h in an incubator at 37 °C. After 1 h, medium was changed. Medium in the wells below inserts was removed with a vacuum aspirator. From cell inserts 200 μL of medium was removed. Both, in the wells below inserts and in cell inserts, 200 μL of medium was added. Plates were returned to the incubator for 1 h. After 1 h, second medium change was performed in the same way, but this time in the wells below inserts, 900 μL of medium was added.

One plate is placed in a standard cell culture incubator in normoxic conditions and other in hypoxic chamber. Medium was changed every 2-3 days during 19 days.

3.5. Total RNA isolation

On the 19. day samples were taken out of the inserts. Medium from wells was removed with a vacuum aspirator while 200 μL was carefully removed from inserts with a pipettor. Scaffolds were washed with PBS which was added both in the inserts and in the wells. After removing PBS, the entire content of each insert is taken out with a sterile spatula and placed in 500 μL of TRIzol® reagent (Invitrogen). Isolation of total RNA is performed according to manufacturer's instructions. In order to lyse cells, samples were homogenized with steel beads for 4 min at 15 Hz followed by addition of 100 μL of chloroform. Samples were thoroughly vortexed and centrifuged for 15 min at 12 000g and 4 °C. Upper aqueous phase, where RNA is located, is transferred to a new tube and 500 μL of isopropanol is added. Samples were vortexed and incubated at -20 °C over night. Next day samples were thawed and then centrifuged for 4 min at 12 000g and 4 °C. Pellet was preserved, mixed with 1 mL of 80% ethanol and centrifuged for 5 min at 20 000g and 4 °C. Ethanol was removed. Pellets of RNA were air dried and diluted in 30 μL of DEPC water in thermoblock at 55 °C for 5 min with mixing. RNA concentration is measured using NanoDrop 1000 spectrophotometer (ThermoScientific). Samples were stored at -80 °C.

3.6. Gene expression analysis by quantitative real-time polymerase chain reaction (qPCR)

Quantitative real-time polymerase chain reaction (qPCR) is a highly sensitive method used for quantification of a specific nucleic acid sequence (DNA, RNA or cDNA) with the detection of PCR product in real time. In this research, qPCR method is used to compare expression levels of cartilage marker genes between samples in order to determine which cell type and culture conditions are most beneficial for chondrogenesis and application in cartilage TE. Expression levels of *SOX9* (which codes for cartilage specific transcription factor) and *ACAN* (aggrecan core protein) were evaluated.

Prior to qPCR, total cell RNA was treated with RNase free DNase I enzyme (BioLabs) according to manufacturer's instructions in order to remove possible contaminations with genomic DNA. Purified RNA was then reverse transcribed in complementary DNA (cDNA). The reverse transcription was performed in 20 µL reaction mixture containing 9,2 µL of DNase treated RNA sample, 4 µL of 25 mM $MgCl_2$ (Fermentas), 2 µL of 10x Buffer without $MgCl_2$ (Applied Biosystems), 2 µL of 10 mM dNTPs (Bio Basic Canada), 0,8 µL of Ribolock RNase inhibitor (Thermo Scientific, 40 U/µL), 1 µL of random hexamers (Invitrogen) and 1 µL of MuLV reverse transcriptase (Applied Biosystems). Reaction mixtures were incubated 10 min at room temperature, 1 h at 42 ℃, 5 min at 99 ℃ and 5 min at 5 ℃. After RT, each sample was diluted with miliQ water to obtain cDNA in a final concentration of 20 ng/µL.

Relative expression of *SOX9* and *ACAN* genes was determined using commercially available primers (Sigma) by quantitative real-time PCR performed on 7500 Fast Real-Time PCR System machine (Applied Biosystems). Endogenous control was *β-actin* housekeeping gene, constitutively expressed in all tissues and cells. Each reaction was performed in a duplicate on a 96-well plate (ABI PRISM Optical 96-Well Plate, Applied Biosystems). Each real-time PCR reaction mixture contained 40 ng of sample cDNA. The detection system was based on SYBR *Green* I flourescent dye (Applied Biosystems) present in qPCR master mix, which binds to double-stranded DNA. As the PCR progresses, more PCR product is created. An increase in flourescence intensity is proportional to the amount of PCR product produced. After real-time PCR reaction, results were analysed using 7500 System Software v2.0.6 and Microsoft Office Excel. For each sample a treshold cycle or Ct value was determined. A Ct value is a cycle number at which the fluorescent signal of the reaction crosses the treshold. A lower Ct value is indicative of higher starting amount of the target in the sample (and vice versa). Ct values were normalized to a Ct value of endogenous control. Fold changes in gene expression were calculated using ΔΔCt method. Since qPCR reactions for samples from both hypoxic and normoxic conditions could not be performed in the same run (on the same 96-well plate), results for samples grown in hypoxic conditions were calibrated to the same reference sample to which results from normoxia were calibrated. On this way, results from both runs could be compared. RQ value for each sample is calculated as a mean value of duplicates. Relative expression levels between samples were compared using Student's *t* test.

3.7. Measuring of dexamethasone release from peptide hydrogel scaffold

Since the concentration of dexamethasone could not be measured in chondrogenic medium and was too low for detection, another system was established where PBS instead of medium was used. Concentration of dexametasone in PBS can be measured more accurately compared to medium. Therefore, two additional peptide hydrogel scaffolds in cell inserts were made.

One contained 100 μL of peptide hydrogel scaffold obtained by mixing 42,5 μL of RADA and equal volume of 20% sucrose with 15 μL of dexamethasone (10^{-2} M stock solution). The final concentration of dexamethasone in the scaffold was $1,5 \times 10^{-3}$ M. Second insert contained only mixture of 50 μL of RADA and 50 μL of 20% sucrose, without dexamethasone. These inserts and wells were filled with only 700 μL of PBS (400 μL in wells and 300 μL in inserts) compared to the 1300 μL of medium which cell culture inserts from the experiment contained. Also, higher concentration of dexamethasone was incorporated within this peptide hydrogel. Smaller volume of PBS and a higher concentration of embbeded dexamethasone resulted in a higher concentration of released dexamethasone which is easier to measure. Every 24 h 2 μL of PBS were taken from inserts and the absorbance was measured at 242 nm using NanoDrop 1000 (Thermo Scientific). Each measurement was repeated 3 x and mean value is calculated. Hydrogel scaffold without dexamethasone was used as a control of hydrogel degradation since absorbance of hydrogel peptide and dexamethasone overlap at 242 nm. The molar extinction coefficient of dexamethasone in PBS at 242 nm is 72 500 $M^{-1}cm^{-1}$ and it was used for calculation of dexamethasone concentration. Concentration was calculated from Lambert-Beer law, where *A=εlc*.

Additionally, 10 μL of dexamethasone (10^{-2} M stock solution) were put in 990 μL of PBS in a 1,5 mL plastic tube, so the final concentration of dexamethasone was 10^{-4} M. This served as a control used to monitor stability of dexamethasone in PBS over time. Every 24 h absorbance was measured at 242 nm.

4. RESULTS

4.1. Morphology of cartilage tissue after chondrogenic dfferentiation

After 19 days of chondrogenic differentiation experiment, morphology of the formed tissue was observed under inverted light microscope (Fig 11). Hydrogel was taken out from the inserts and put in TRIzol reagent for RNA isolation afterwards (Fig 12).
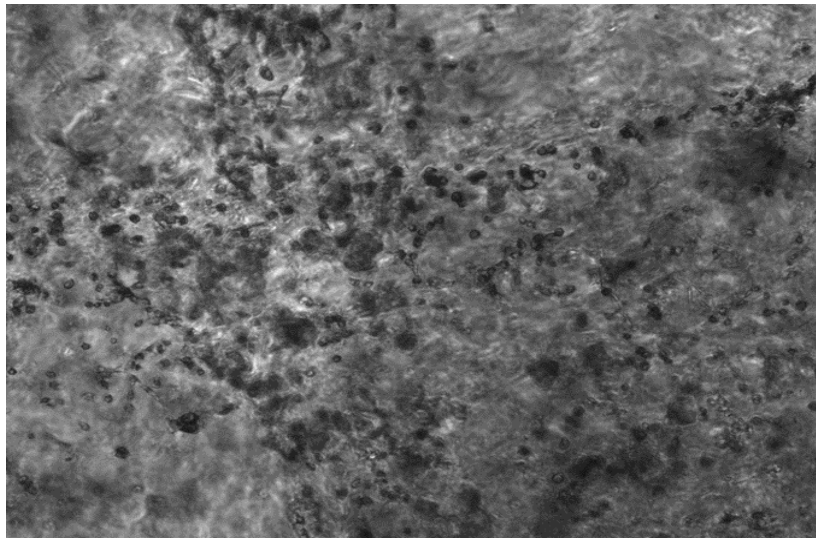
Figure 11. Morphology of cartilage tissue in the peptide hydrogel scaffold after 19 days of chondrogenic differentiation. Combination of chondrocytes and hBMSCs grown in hypoxic conditions with dexamethasone incorporated within the peptide hydrogel scaffold. Photograph taken under inverted light microscope, 50x.

Peptide hydrogel

Figure 12. Appearance of cell inserts after 19 days (left). Transparent layer of peptide hydrogel is visible at the bottom of the insert; the rest is chondrogenic medium. **Peptide hydrogel taken out of the cell insert (right).**

4.2. Kinetics of dexamethasone release from the peptide hydrogel scaffold

Dexamethasone was mixed with the peptide hydrogel and became entrapped within during the gelation process. As hydrogel degraded, dexamethasone released in PBS and its absorbance was measured with spectrophotometer at 242 nm. In the first 24 h, there was a rapid increase of dexamethasone concentration, when 30% of dexamethasone was released from the peptide hydrogel. From day 2 to day 8, concentration did not change considerably, while from day 8 to day 12 additional 30% of dexamethasone was released. The majority of hydrogel was degraded around day 12 since after day 12 dexamethasone concentration no longer changed (Fig 13).
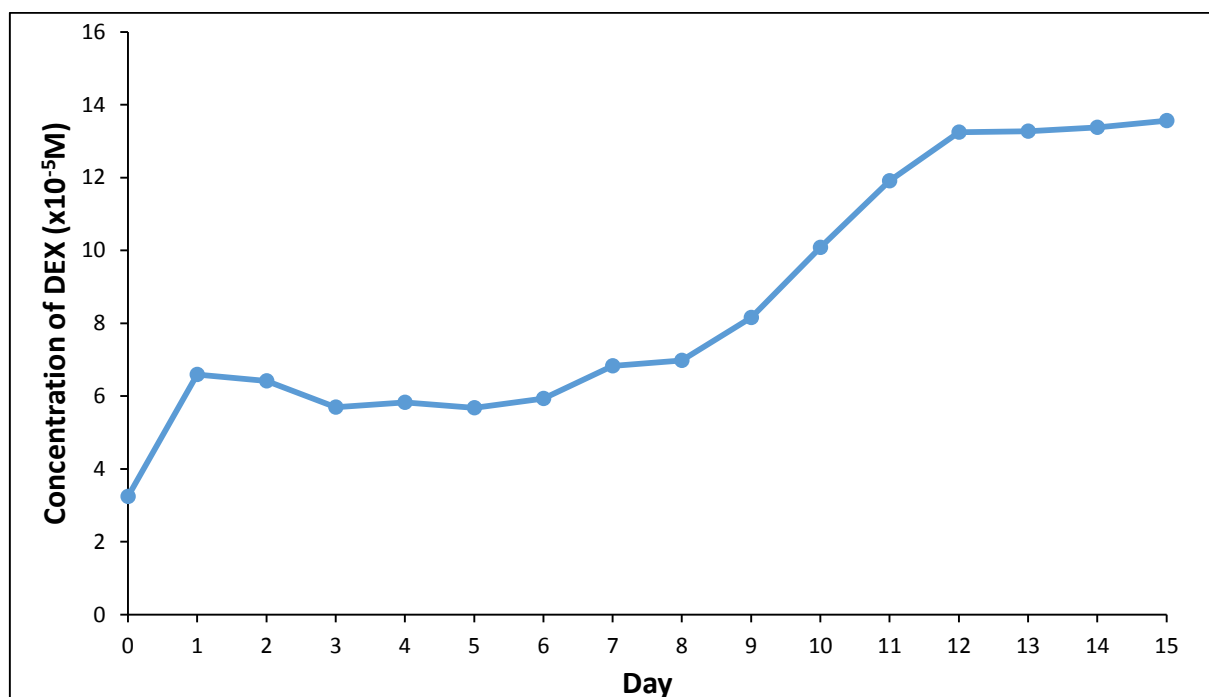
Figure 13. Cumulative release of dexamethasone from peptide hydrogel scaffold in cell insert. The initial concentration of dexamethasone in the peptide hydrogel was 1,5 x10^{-3} M.

These results were used for approximation of dexamethasone release from the peptide hydrogel scaffold in the 3D system established for cartilage TE. The initial concentration of dexamethasone in these scaffolds was 1,5 x10^{-5} M.
The volume of chondrogenic medium was 1300 µL. Medium changes were performed every 2-3 days in the period of 19 days (Fig 14).
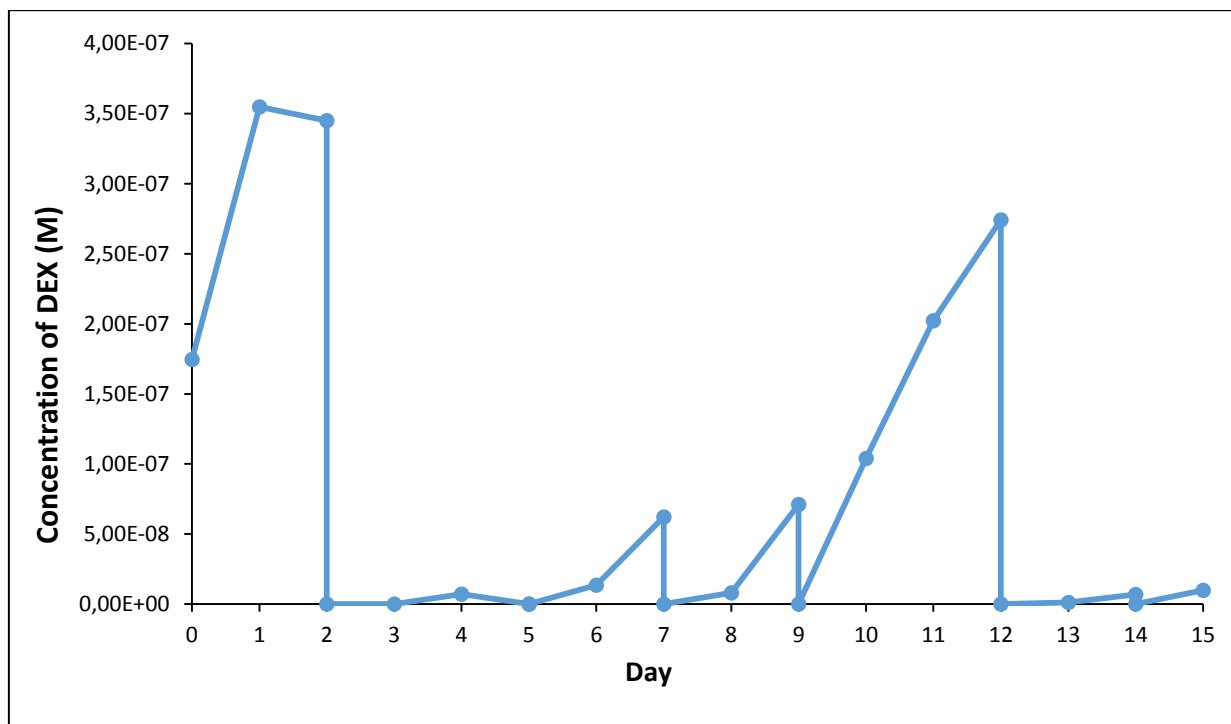
Figure 14. Concentration of dexamethasone in the chondrogenic medium in cell inserts where dexamethasone was incorporated within the peptide hydrogel (1,5 x 10⁻⁵M).
Sudden decreases of concentration to 0 (vertical lines) represent the time when chondrogenic medium was changed.

In the first 2 days, dexamethasone concentration in chondrogenic medium was between $1,75 \times 10^{-7}$ M and $3,55 \times 10^{-7}$ M. After first medium change, from day 2 to day 6, concentration was very low. At day 7 and 9, concentration was around 6×10^{-8} M. From day 9 to day 12, concentration considerably increased and cells were exposed to a dexamethasone concentration in the range from $1-2 \times 10^{-7}$ M. After medium change at day 12, concentration remained significantly low until the end chondrogenic differentiation experiment.

4.3. Expression of cartilage marker genes

Relative expression of cartilage marker genes *SOX9* and *ACAN* was determined at mRNA level using qPCR. RNAs isolated from bone and native cartilage were used as a negative and positive control, respectively. Relative expression of *SOX9* and *ACAN* in all samples was calibrated to the same reference sample- combination of chondrocytes and hBMSCs grown in normoxic conditions with dexamethasone added in the chondrogenic medium. The results showed that chondrogenic differentiation of various cell types was not equally successful. Chondrocytes turned to have the highest chondrogenic potential among used cell types (Fig 15). Relative expression of *SOX9* and *ACAN* in hBMSCs compared to other cell types was extremely low (Fig 15, 16, 17 and 18). Therefore, hBMSCs showed the lowest chondrogenic potential. Considering the influence of oxygen tension on chondrogenic differentiation, only in the case of chondrocytes there was a statistically significant difference, where hypoxic conditions proved to be more beneficial for chondrogenesis than normoxic conditions (Fig 15). Dexamethasone source, in chondrogenic medium or peptide hydrogel scaffold, did not significantly influence on differences in *SOX9* and *ACAN* expression levels between samples.

Figure 15. Relative expression of *SOX9* gene in samples of chondrocytes, hBMSCs and their combination grown in hypoxic (H) and normoxic (N) conditions. Dexamethasone was added in the chondrogenic medium. *p<0,05

Figure 16. Relative expression of *SOX9* gene in samples of chondrocytes, hBMSCs and their combination grown in hypoxic (H) and normoxic (N) conditions. Dexamethasone was incorporated within the peptide hydrogel scaffold.

Figure 17. Relative expression of *ACAN* gene in samples of chondrocytes, hBMSCs and their combination grown in hypoxic (H) and normoxic (N) conditions. Dexamethasone was added in the chondrogenic medium.
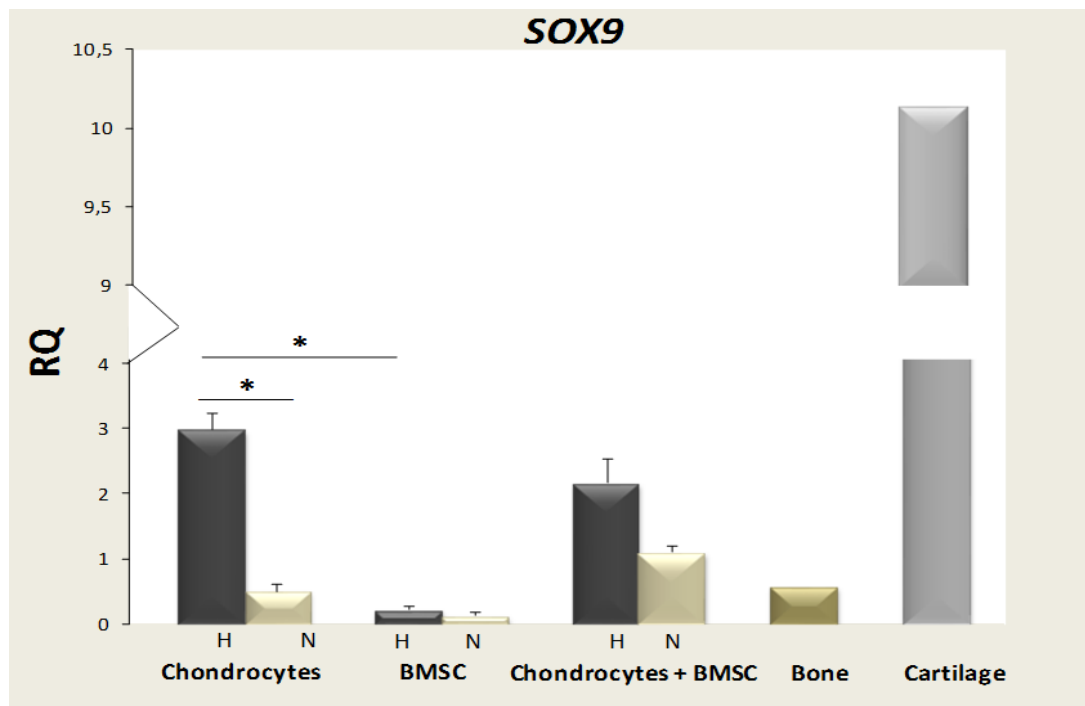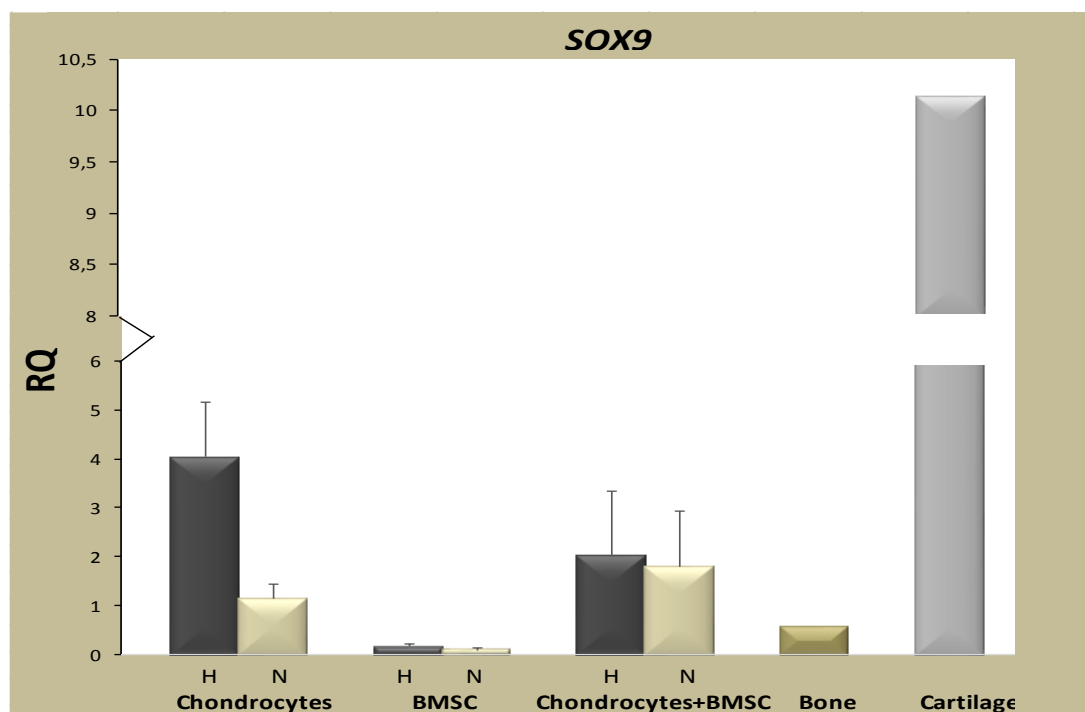

Figure 18. Relative expression of *ACAN* gene in samples of chondrocytes, hBMSCs and their combination grown in hypoxic (H) and normoxic (N) conditions. Dexamethasone was incorporated within the peptide hydrogel scaffold.
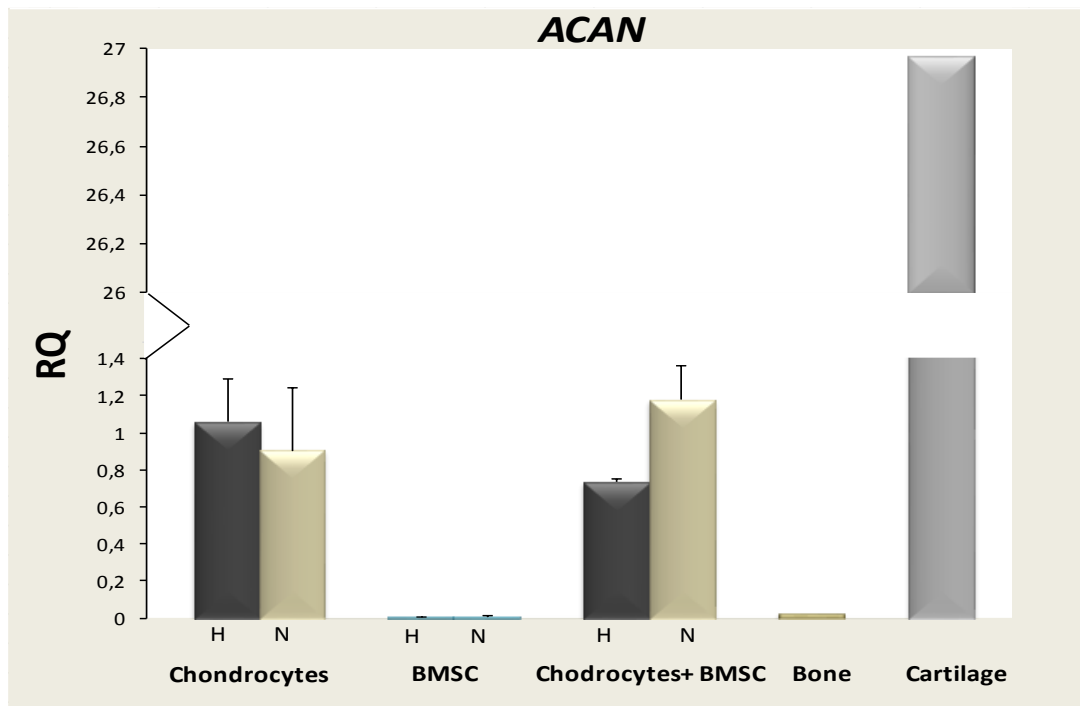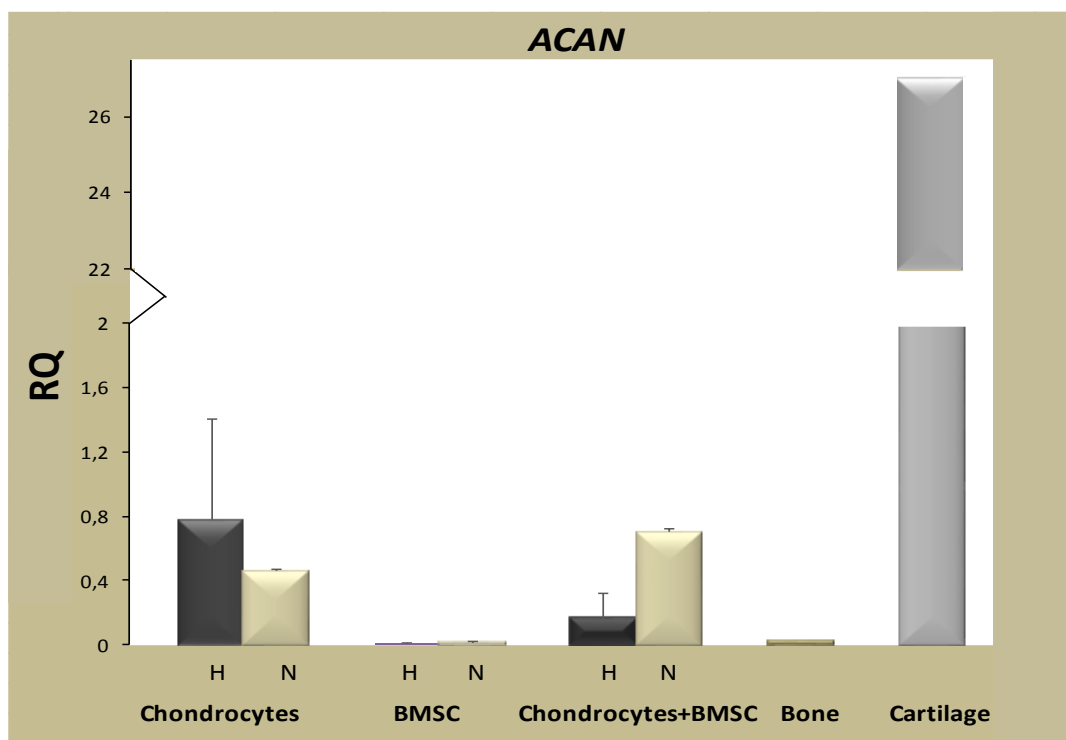
5. DISCUSSION

Articular cartilage is a tissue with very limited ability for self-repair. This is mostly attributed to the absence of blood vessels, nerves and undifferentiated progenitor cells that can promote repair process. Common methods for treatment of cartilage defects and injuries are not entirely effective, resulting in a formation of tissue which is structurally and functionally inferior to native articular cartilage and often involve invasive procedures that require long period of rehabilitation. With an aging population and growing problem of obesity, it is assumed that cartilage injuries and defects will become major health, social and economic problem in the near future. Tissue engineering as a new and revolutionary field of medicine could provide improvements and solutions in treatment and repair of many damaged tissues and organs, including articular cartilage. Tissue engineering combines cells, 3D scaffolds and appropriate inductive signals (chemical, mechanical, physical) to produce a functional tissue construct that can be implanted in a patient in order to restore or improve structure and function of damaged/injured tissue. This approach opens a possibility of using patient's own cells to overcome problems with transplant rejection or disease transmission. The main challenge of tissue engineering is to choose a right combination of cells, scaffold, chemical signals and conditions that will finally result in a formation of desired tissue. This is also a great challenge in cartilage tissue engineering.

In this respect, the aim of this study was to optimize conditions for 3D *in vitro* chondrogenesis. The optimization method included: *i)* cell type, *ii)* oxygen tension and *iii)* source of dexamethasone. Chondrogenic potential of three different cell types was analyzed: chondrocytes, human bone marrow mesenchymal stem cells (hBMSCs) and co-culture of chondrocytes and hBMSCs in 2:1 ratio. Cells were grown in a 3D system in cell inserts, encapsulated in a self-assembling peptide hydrogel (RADA)$_4$, a new class of scaffold widely researched for the purposes of tissue engineering. Cells were grown in a chondrogenic medium for 19 days. One of the chondrogenic medium components, dexamethasone, was added in a chondrogenic medium or was incorporated with cells within a peptide hydrogel scaffold. In order to explore the effects of oxygen tension on chondrogenic differentiation, cells were grown in either hypoxic (5% O_2) or normoxic (20% O_2) conditions. After 19 days, samples were harvested and expression of two cartilage marker genes *SOX9* and *ACAN* was analyzed using qPCR. Additionally, kinetics of

dexamethasone release from the peptide hydrogel was explored in order to determine dexamethasone concentration to which cells were exposed during period of 19 days.

According to the results of gene expression analysis, chondrocytes grown in hypoxic conditions showed the highest chondrogenic potential among used cell types. Before chondrogenic differentiation experiment, chondrocytes were expanded in a monolayer culture in order to obtain sufficient cell number for seeding in a peptide hydrogel. Several studies showed extensive changes in gene expression in chondrocytes upon passaging. This process mainly affects collagen II production and genes involved in signaling networks responsible for maintenance of chondrocyte's phenotype. Dedifferentiation represents the main obstacle for cartilage tissue engineering using chondrocytes as a cell source (Ma et al, 2013; Schnabel et al, 2002). However, this process is reversible. Caron et al (2012) investigated the effects of 2D and 3D culture system on redifferentiation of passaged chondrocytes. Three-dimensional cultures expressed cartilage specific genes for collagen type II, aggrecan core protein and SOX9 transcription factor, while 2D did not. Furthermore, a reduced oxygen tension also proved to promote redifferentiation of passaged chondrocytes. Chondrocytes grown in hypoxic conditions (2-5% O_2) showed enhanced production of ECM components (collagen type II, aggrecan, GAGs) and decreased production of collagen type I, collagen type X, matrix metalloproteinases and aggrecanases (Markway et al, 2013; Coyle et al, 2009; Murphy & Sambanis, 2004). Therefore, based on these studies and obtained results, it can be assumed that RADA encapsulation and reduced oxygen tension helped restore key differentiated phenotypic markers of passaged chondrocytes.

Human bone marrow mesenchymal stem cells showed the lowest chondrogenic potential in this study. Expression levels of *SOX9* and *ACAN* genes were extremely low regardless of oxygen tension and there was no difference between samples grown in normoxia and hypoxia. This result is inconsistent with most of the studies involving hBMSCs chondrogenesis where oxygen tension proved to be important promoting factor. In most of the studies, hypoxia proved to enhance proliferation and chondrogenic differentiation of hBMSCs, which was also expected here (Ren et al, 2006; Robins et al, 2005). Grayson et al (2006) reported an increase in osteogenic and adipogenic differentiation of 3D cultures of bone marrow derived mesenchymal stem cells under

hypoxia. Compared to this study, in Grayson's experiment cells isolated from bone marrow were both expanded and differentiated in 3D architecture under hypoxic conditions. In this study, hypoxic conditions and 3D culture system were applied only during the differentiation experiment. Cicione et al (2013) also applied hypoxia only during *in vitro* directed differentiation of hBMSCs. In their study, chondrogenic differentiation also was absent in severe hypoxic conditions. As known from the literature, hypoxia can act as a stimulus altering cellular metabolism through the activity of hypoxia-inducible factor-1 α (HIF-1α) (Semenza, 2001). It is shown that HIF-1α downregulates mitochondrial oxygen consumption and upregulates key markers of stem cells like Oct-4 (D'Ippolito et al, 2006). Cicione and colleagues hypothesized that relationship between HIF-1α, cell proliferation and stem cell markers could explain the absence of stem cell differentiation under hypoxic conditions. Dos Santos et al (2010) reported that hypoxia promotes cell proliferation and expansion. During proliferation, the majority of the cells undergo cell division and cannot begin the cellular mechanisms involved in differentiation. Therefore, expansion of hBMSCs in hypoxia seems to promote proliferation and preserve their differentiation capacity (so called 'stemness'). For further optimization of cartilage tissue engineering using hBMSCs, both expansion and *in vitro* differentiation are recommended to be carried out in hypoxic conditions.

This, however, does not explain a failure of hMBSCs grown in normoxic conditions to undergone chondrogenesis. Expression levels of *SOX9* and *ACAN* were as low as in hBMSCs grown in hypoxic conditions. Xu et al (2008) characterized temporal changes in expression of chondrogenic genes and developed a staging scheme for *in vitro* chondrogenic differentiation of hMSCs grown in 3D alginate-gels. Using qRT-PCR, they demonstrated a largely characteristic temporal pattern of chondrogenic markers and divided the progression of cellular phenotype into four main stages. According to their results, *SOX9* expression reaches peak level in stage II (days 6-12), while *ACAN* attains its peak levels in stage IV (days 18-24). Therefore, *ACAN* as a late marker of chondrogenic differentiation of hBMSCs, should have high(er) expression level at day 19. But since both genes showed negligible expression levels, it brings to conclusion that chondrogenic differentiation in hBMSCs grown both in hypoxic and normoxic conditions was not induced.

Co-culture of chondrocytes and hBMSCs in 2:1 ratio proved to have higher chondrogenic potential than hBMSCs, but lower than chondrocytes. Oxygen tension did not significantly influence on chondrogenesis in co-culture, although samples grown in normoxia demonstrated higher expression levels of *ACAN* compared to samples grown in hypoxia. Cheng et al (2011) demonstrated that co-culture of rabbit chondrocytes and rabbit BMSCs at defined ratios can promote the expression of cartilaginous ECM. They determined the 2:1 ratio (chondrocytes:BMSCs) to be optimal since the expression of collagen type II and aggrecan in this group on day 21 was higher than in other groups containing individually cultured chondrocytes and BMSCs or co-culture of chondrocytes and BMSCs in 4:1, 1:1, 1:2 and 1:4 ratio. Neocartilage can develop through co-culturing of chondrocytes and hBMSCs even in the absence of biomolecular factors such as serum and exogenous growth factors. It is hypothesized that chondrocytes promote chondrogenesis of BMSCs via paracrine regulation since they produce and secrete a variety of protein molecules including TGF-β, IGF-1, FGF-2, BMP-2, and many other inductive factors that still need to be determined (Yang et al, 2012). Using the co-culture approach, Cooke et al (2011) demonstrated neocartilage formation without hypertrophy and calcification.

Since individually cultured hBMSCs showed much lower chondrogenic potential than co-culture, it can be assumed that chondrocytes in the co-culture enhanced chondrogenesis of hBMSCs in this study. Co-culturing of chondrocytes and BMSCs is a novel and promising approach in cartilage tissue engineering, but requires further research and optimization.

Analysis of kinetics of dexamethasone release from the peptide hydrogel scaffold showed that in the first 24 h, 30% of dexamethasone was released. This initial burst is most likely caused by *i)* molecules that were at or near medium-hydrogel interface and escaped rapidly into the medium and *ii)* dexamethasone release that happens faster from the larger pores of hydrogel. After 24 h, a plateau value is reached. From day 9 to day 12, another significant increase in concentration happened, while after day 12 the majority of hydrogel was degraded and another plateau is reached. Until day 15, about 63% of dexamethasone was released. Koutsopoulos et al (2009) demonstrated that in hydrogel systems drug or protein release rarely reaches 100%. The main reason is physical entrapment of molecules in highly entangled nanofiber domains of the hydrogel. Since hydrogels are biodegradable, an introduction of hydrogel into a living organism will cause

hydrogel to degrade entirely. This process will allow to the rest of the load from peptide hydrogel to be released in the surrounding tissue. Therefore, this study shows that self-assembling peptide hydrogel RADA may be useful as a carrier for therapeutic proteins for sustained release applications.

Dexamethasone source did not have significant influence on the differences in *SOX9* and *ACAN* expression levels between samples in this study. In most studies, chondrogenic culture medium is supplemented with 100 nM dexamethasone over a multiweek culture period. Approximation of dexamethasone release from peptide hydrogel in the medium in which cells were grown showed that in the first 10 days, from day 2 to day 9, and after day 12, dexamethasone concentration was lower than recommended for chondrogenic differentiation, and still, expression levels of chondrogenic markers in samples where dexamethasone was embedded in the hydrogel were not significantly lower than in samples where dexamethasone was added in the medium in a constant optimal concentration. This is consistent with some recent studies which suggest that even lower concentration of dexamethasone can still induce chondrogenesis. Tangtrongsup & Kisiday (2015) compared the influence of 100 nM and 1 nM dexamethasone on chondrogenesis of equine MSCs encapsulated in agarose. The ECM accumulation was not significantly different in 100 nM and 1 nM dexamethasone, although it was suppressed by ~40% in dex-free samples. One nanomolar dexamethasone however, did not prevent undesired hypertrophic differentiation. Similarly, Enochson et al (2012) recommend 50 nM dexamethasone. Tangtrongsup and colleagues also explored the effect of timing of exposure to dexamethasone. Early timing of exposure seems not to be critical, while sustained exposure of at least one week appears to be necessary to maximize ECM accumulation. Shintani & Hunziker (2011) hypothesized that a final effect of dexamethasone on MSCs depends on their tissue origin and a nature of used growth factors. Therefore the effects of dexamethasone and its role in chondrogenesis need to be more explored and explained.

Although great advances in the field of cartilage tissue engineering were made, an ideal protocol regarding cell source, scaffold, biochemical and physical factors is still not established. One of the main challenges still remains unsolved: to produce functional construct that mimics a complex structure and zonal organization of cartilage.

6. CONCLUSION

Based on the obtained results, it can be concluded:

- Chondrocytes under hypoxic conditions have the best potential for cartilagenous tissue formation. Hypoxic conditions, 3D system for *in vitro* differentiation and addition of chondrogenic medium can induce redifferentiation of passaged chondrocytes.

- Bone marrow-derived mesenchymal stem cells have the lowest chondrogenic potential.

- If hypoxic conditions are not available, the combination of chondrocytes and hBMSCs in 2:1 ratio is recommended for cartilage formation.

- Source of dexamethasone does not have a significant infleunce on efficiency of *in vitro* chondrogenesis.

- Kinetics of dexamethasone release from peptide hydrogel RADA follows the kinetics of hydrogel degradation over time. Since RADA is biocompatible and biodegradable, it may be useful as a carrier for therapeutic proteins and other molecules for sustained release applications.

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CURRICULUM VITAE

I was born on 10 of August 1991. in Brčko, Bosnia and Herzegovina. I attended primary school in Posavski Podgajci and general-education secondary school in Županja. In 2010 I started the study of Molecular Biology at Faculty of Science, University of Zagreb. In 2013 I finished undergraduate and started graduate study of Molecular Biology.

 I have participated in an event "Night of Biology" traditionally organized by Biology Department every year: first time in 2012 in a workshop about endoplasmatic reticulum and then in 2015 in a workshop about UV radiation and its potential harmful activity. In 2013, I attended Laboratory Skill Training at Ruđer Bošković Institute, Division of Molecular Biology, in Laboratory for Molecular and Cellular Biology under supervision of Marija-Mary Sopta, Phd. As a student volunteer, I have participated in organization of 9th ISABS conference on forensic and anthropologic genetics and Mayo clinic lectures in individualized medicine which took place in Bol, Island of Brač, Croatia in June 2015 organized by International Society for Applied Biological Sciences and Mayo clinic. I also participated in the 3rd International Conference on Regenerative Orthopaedics and Tissue Engineering (ICRO) in November 2015 with a poster presentation under the title: "Optimization of conditions for three-dimensional *in vitro* cartilage growth". In 2015, I was awarded with stipend for excellence of the University of Zagreb.

I was interested in science since primary school. I grew up in a village in Slavonija, surrounded by nature and animals which evoked my curious, exploratory spirit. My great passion in that time was also astronomy and everything that was somehow connected to space. In secondary school I became more interested in chemistry, biology, magnificent complexity and organization of human body and all living forms that surround us.

My current interests include: epigenetics, stem cells, biology of tumors, immunology, microbiology. I prefer unusual and unconventional approaches to scientific problems.